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Strain-Specific Antigens of *Toxoplasma gondii*

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A detailed immunological assessment of strain-specific antigens of *Toxoplasma gondii* has not been reported. We developed rabbit antisera against three strains of toxoplasma obtained from divergent sources. These strains included the frequently studied laboratory strain RH, strain C, obtained from a naturally infected kitten, and strain P, which is maintained by passage in mice. The rabbit antisera were used to identify unique strain-specific and commonly shared tachyzoite antigens by radioiodination followed by immunoprecipitation and Western blot analysis. Both qualitative and quantitative differences of a number of the major tachyzoite antigens were found in these assays. A parasite plaque reduction assay using parasiticidal monoclonal antibody showed marked differences in the ability to kill these three different tachyzoite subtypes, further supporting antigenic variation among *T. gondii* strains.

Toxoplasma gondii is an infectious pathogen of worldwide distribution that causes toxoplasmosis. Although morphologic and antigenic variation among the life cycle stages are recognized (bradyzoite, tachyzoite, and oocyst-sporozoite), there has been no immunologic classification regarding the various strains of *T. gondii* (1, 4, 9, 12, 14). Most strains have been defined as either virulent or less virulent depending on their morbidity and mortality in toxoplasma-infected mice (13). By far, the most commonly used virulent strain of *T. gondii* is RH. This strain, originally obtained from infected human tissue, has been maintained in the laboratory since 1941 by continuous passage in either mouse peritoneal cavities or cultured human fibroblasts (HF) (18, 21). Over the years, many investigators have found this strain to be highly virulent in susceptible mice, frequently with a 90% lethal dose of <100 viable organisms (13, 18). Because of its aggressive nature and ease of growth both in vivo and in vitro, it has been the predominantly studied strain of *T. gondii*. RH strain toxoplasma has been shown repeatedly to produce neither tissue cysts containing bradyzoites nor oocyst-sporozoites, the parasite excreted after sexual reproduction in cat intestines (3). We have reported on in vitro selection of a monoclonal antibody-resistant antigenic mutant of *T. gondii*. This RH strain variant was found to be devoid of the major surface membrane protein P22 (10). Other, less virulent strains of *T. gondii* have been studied in the past to various ends (5, 6, 21). Some reports have tried to define antigenic strain variation by immunological means. Suggs et al. (21) compared four strains of *T. gondii* with two *Besnoitia* strains by using several immunologic tests, such as immunofluorescence and complement fixation. They concluded that there are strain variations possibly brought on by prolonged passage in mice. In an attempt to correlate virulence with strain variation, Handman and Remington infected mice with either the virulent RH strain or the less virulent C37 and C56 strains of *T. gondii* and characterized the antibodies that developed (6). By radioiodination and immunoprecipitation, they concluded that antisera to these strains recognized similar membrane antigens.

In this study, we demonstrated that three divergent *T. gondii* strains exhibit strain-specific antigenic variability. This strain variance can be detected by several different but highly specific immunologic tests, including Western blotting and parasite plaque reduction with monoclonal antibody.

MATERIALS AND METHODS

Parasites and host cells. The methods for growth and cloning of *T. gondii* in cultured HF have been previously described (17). The three strains of tachyzoites used in these experiments were cloned lines of strain RH, P, or C. The RH strain tachyzoite has been maintained in our laboratory for many years by continuous passage in HF. P strain parasites were obtained from the Me49 line of *T. gondii* from M. Lunde, National Institutes of Health, Bethesda, Md., and cloned in our laboratory. P strain tachyzoites were produced by infecting HF monolayers with P strain-infected mouse brain that had been homogenized and forced through a 27-gauge needle. These were maintained as tachyzoites by continuous passage in HF rather than standard passage in mice. C strain tachyzoites were cloned and used to infect a kitten, and the resulting oocysts were collected. C strain tachyzoites were then obtained by mechanical fracturing of oocysts followed by subculturing of sporozoites in HF. All experiments involving C and P strain tachyzoites were carried out between passages 1 and 30 of the tachyzoite parasites in culture. Extracellular tachyzoites were obtained in a manner similar to that previously described (9). Briefly, tachyzoites were harvested from infected monolayers by forced extrusion through a 27-gauge needle. Residual host cell debris was removed with phytohemagglutinin (7).

Rabbit antisera. Antisera to the three strains of *T. gondii* were raised in adult New Zealand White rabbits. Preimmunized serum was taken for a normal control. The rabbits for each parasite strain were immunized intradermally with a total of 7.5×10^8 freeze-thaw-killed tachyzoites in three doses. The first injection was given with Freund complete adjuvant, and the remaining two were given with Freund incomplete adjuvant. Rabbits were bled 10 days after the last injection.

ELISA. Antitoxoplasma antibody was measured with an enzyme-linked immunosorbent assay (ELISA) that has been previously described (10). Purified parasites were placed in microtiter plates, dried overnight, and blocked with 5% fetal bovine serum–phosphate-buffered saline (PBS). Antisera were incubated for 2 h at 37°C, and the plates were washed in 0.05% Tween 20–PBS. Antibody binding was identified with peroxidase-labeled goat anti-rabbit immunoglobulin G

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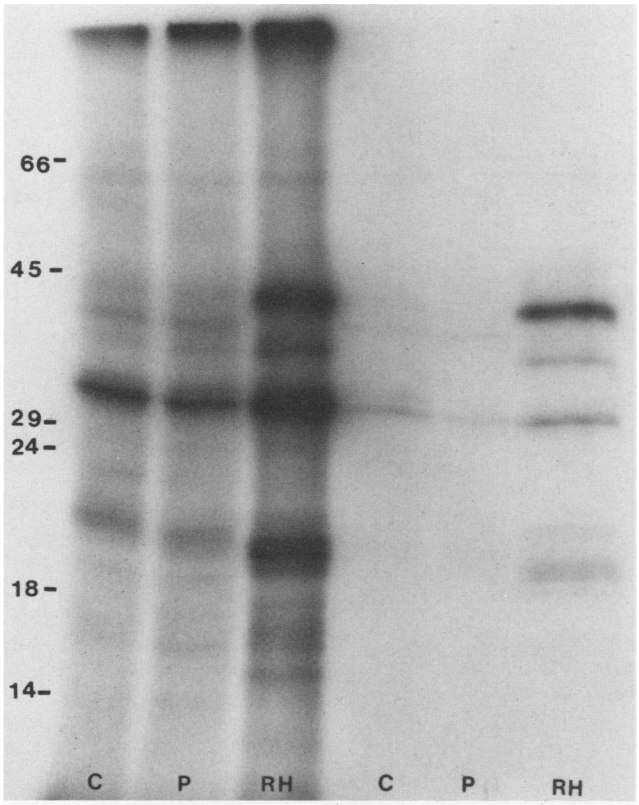

FIG. 1. Autoradiographic comparison of SDS-PAGE of radioiodinated and immunoprecipitated tachyzoites of three *T. gondii* strains. Equal numbers of (5×10^7) of tachyzoites of each strain were purified and iodinated in parallel by the Iodogen method. The left three lanes show equal numbers of parasitesof each radiolabeled strain. The right three lanes show immunoprecipitation of equal numbers of radioiodinated *T. gondii* tachyzoites with strain-specific rabbit antisera. All samples were run on a 12.5% polyacrylamide gel, dried, and autoradiogrammed for 2 weeks.

(IgG) (Boehringer Mannheim Biochemicals) or IgM (Cooper Biomedical, Inc.).

SDS-PAGE. Parasite samples were dissolved in sample buffer that contained 2% sodium dodecyl sulfate (SDS) and 5% 2-mercaptoethanol. After boiling for 3 min, samples were electrophoresed in a discontinuous 0.1% SDS–12.5% polyacrylamide gel (SDS-PAGE). Molecular weight standards included β-galactosidase (116,000), phosphorylase *b* (97,000), bovine albumin (66,000), egg albumin (45,000), carbonic anhydrase (29,000), trypsinogen (24,000), β-lactoglobulin (18,400), and lysozyme (14,300).

Nitrocellulose blot transfer electrophoresis. A modification of the method of Towbin et al. was used (22). After SDS-PAGE, gels were applied to a sheet of 0.2-μm (pore size) nitrocellulose paper and electrophoresed for 2 h with a Transfor apparatus (Hoefer Scientific). After electrophoresis, the remaining protein-binding sites on the nitrocellulose paper were blocked with 3% bovine serum albumin in PBS, pH 7.4, overnight at 4°C. Strips were incubated with appropriate antisera diluted 1:500 in 3% bovine serum albumin–PBS for 2 h at room temperature. The nitrocellulose was washed several times in 0.05% Tween 20–PBS and incubated with diluted peroxidase-labeled goat anti-rabbit IgG (1:6,000) or IgM (1:1,000). The strips were washed and developed in a solution of 0.02% 4-chloro-1-naphthol–0.1% hydrogen peroxide in PBS. The reaction was stopped by washing with H_2O. Strips were stored in the dark until photographed.

Radioiodination of proteins and immunoprecipitation of *T. gondii* antigens. Surface proteins of *T. gondii* were iodinated by the Iodogen technique as previously described (11). After iodination, proteins were dissolved in 0.5% Nonidet P-40 nonionic detergent for 60 min at 4°C. Immunoprecipitation of *T. gondii* antigens was done in a manner similar to that previously described (9). Briefly, 10 μl of radioiodinated *T. gondii* detergent extract was precleared with 25 μl of a 25-mg/ml suspension of protein A–Sepharose Cl-4B (Sigma Chemical Co.), mixed with 10 μl of rabbit antiserum and incubated for 1 h at 37°C. To this mixture was added another 25 μl of protein A–Sepharose Cl-4B. This was allowed to incubate for 1 h at 4°C. The suspension was then pelleted and washed twice with 0.5% Nonidet P-40 in PBS and twice in 0.05 M Tris (pH 8.3)–0.5% Nonidet P-40–1 M NaCl. The final pellet was suspended in 2× sample buffer and electrophoresed as usual.

Monoclonal antibodies and plaque reduction assay. We have previously reported the development of hybridomas that secrete monoclonal antibodies that react with tachyzoites of *T. gondii* (10, 11). Biologic activity of the antibodies was determined by a plaque reduction assay. This is a parasiticidal test based on complement-mediated killing of parasites. Extracellular tachyzoites (10^5) of each of the three strains were incubated for 90 min at 37°C with 10 μl of monoclonal antibodies–50 μl of normal human serum previously shown to be nonparasiticidal in a total volume of 100 μl of tissue culture medium. Control parasites were treated with control P3 ascites (an IgG myeloma protein unrelated to *T. gondii* produced by P3-X63-Ag8) replacing toxoplasmacidal monoclonal antibody. Viable parasites were determined by plaque formation in HF.

RESULTS

ELISA of strain-specific rabbit antiserum. To determine whether *T. gondii* tachyzoite antigens vary in their immunogenicity, we produced rabbit antiserum to each of the parasite strains under investigation. Rabbits were immunized with killed parasites as described in Materials and Methods. Antitoxoplasma antibody IgG titer was determined by ELISA at >1:96,000 for each of the three rabbit antisera. A similar ELISA was performed to determine IgM titer. By this test, the RH strain IgM titer was threefold less than that of the C and P strains (1:1,350). To determine cross-reacting antibody titer, we tested each of the antisera by ELISA against each of the strains. For this assay, equal numbers of RH, C, and P strain tachyzoites (5×10^5 parasites per well) were used as the antigenic substrate and assayed for IgG binding. All three rabbit antisera had a titer of >1:96,000 against each of the parasite strains. Thus, it was not possible to detect strain specificity in the antisera by the ELISA.

Radioiodination and immunoprecipitation with strain-specific rabbit antiserum. To evaluate strain variation biochemically, we performed radioiodination studies. Equal numbers of tachyzoites of the three strains were purified and iodinated in parallel. Figure 1 shows the results of an autoradiogram of a 12.5% gel run with equal numbers of parasites of the RH, P, and C strains. Our results are consistent with previously published observations by others showing that the major iodinatable surface proteins are shared (5, 6). These proteins are of approximate molecular

weights of 22,000, 30,000, 37,000, and 43,000 (designated P22, P30, P37, and P43). Although the P43 and P22 bands were present on all three strains,they were quantitatively reduced in the P and C strains. Strains P and C also shared a protein of an approximate molecular weight of 40,000 (P40) that was not present on strain RH. An autoradiogram run under identical conditions but with an equal number of counts showed the same results (data not shown).

To determine the specific parasite protein antigens recognized by the rabbit sera, equal numbers of iodinated parasites of each of the three strains were immunoprecipitated by using their corresponding antisera. Figure 1 also shows the results of this experiment. All three rabbit antisera precipitated P30. Additionally, the P and C antisera precipitated P40 that did not appear in the RH iodination pattern. Conversely, RH antiserum precipitated P43 and P37, which were not precipitated by P and C strain antisera.

Western blot analysis of proteins. Western blot analysis was performed to identify other unique strain-specific tachyzoite antigens. To determine whether these strain-specific antisera react with host cell antigens, we used the same parasite purification procedure with uninfected HF. Parasites (3×10^6 per lane) and an equivalent volume of detergent-soluble HF were electrophoresed on a 12.5% polyacrylamide gel and transferred to nitrocellulose, strips of which were incubated with strain-specific rabbit antiserum and assayed for IgG binding (Fig. 2). The strips on the left are parasite antigens, while those on the right are HF antigens. There was no significant binding to HF antigens.

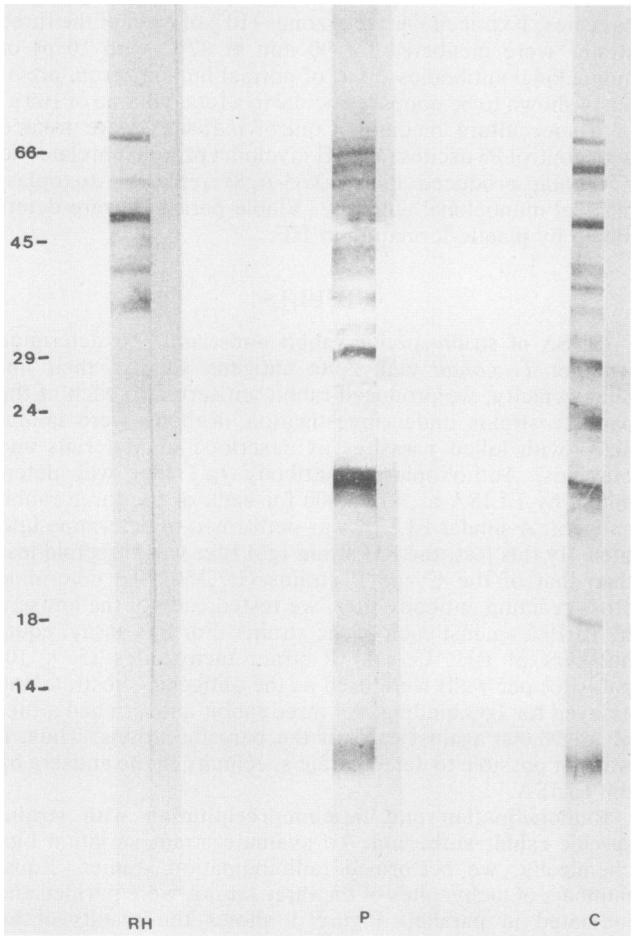

FIG. 2. Nitrocellulose blot transfer of 3×10^6 RH, P, and C strain tachyzoites and an equivalent volume of purified HF antigens reacted with strain-specific rabbit antisera and assayed for IgG binding. Each left lane contained purified tachyzoite, and each right lane contained HF antigens.

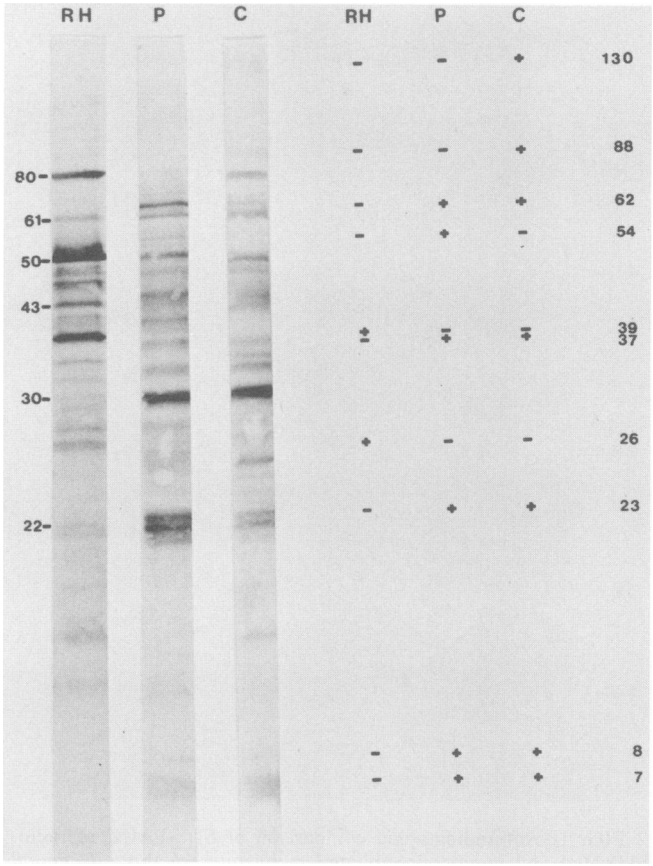

FIG. 3. Western blot analysis of RH, P, and C strain antigens reacted with strain-specific rabbit antisera. Equal numbers (3×10^6) of purified tachyzoites from strains RH, P, and C were subjected to 12.5% polyacrylamide gel and electrophoretically transferred to nitrocellulose paper, strips of which were incubated with corresponding antisera and assayed for IgG binding. The column of numbers on the left indicates antigens of the same molecular weight recognized by all three antisera. The column of numbers on the right indicates the molecular weights of unique or partially shared parasite antigens.

To determine strain-specific antigens, equal numbers of RH, P, and C parasites (3×10^6 per lane) were electrophoresed on a 12.5% polyacrylamide gel transferred to nitrocellulose, strips of which were incubated with the corresponding strain-specific rabbit antisera and assayed for IgG or IgM binding. Figure 3 shows those antigens recognized by the IgG antibody fraction. Numerous antigens of similar molecular weights were identified in all three strains. This includes major immunogenic determinants at P80, P61, P50, P43, P30, and P22 (marked in the far left lane). Although P30 and P22 were readily iodinated and immunoprecipitated with RH antisera, the Western blot showed a marked reduction in reactivity to these antigens. To determine whether this was an individual antibody response in the rabbit rather than a parasite strain variation, antiserum from a second rabbit immunized with RH parasites was used in a Western blot

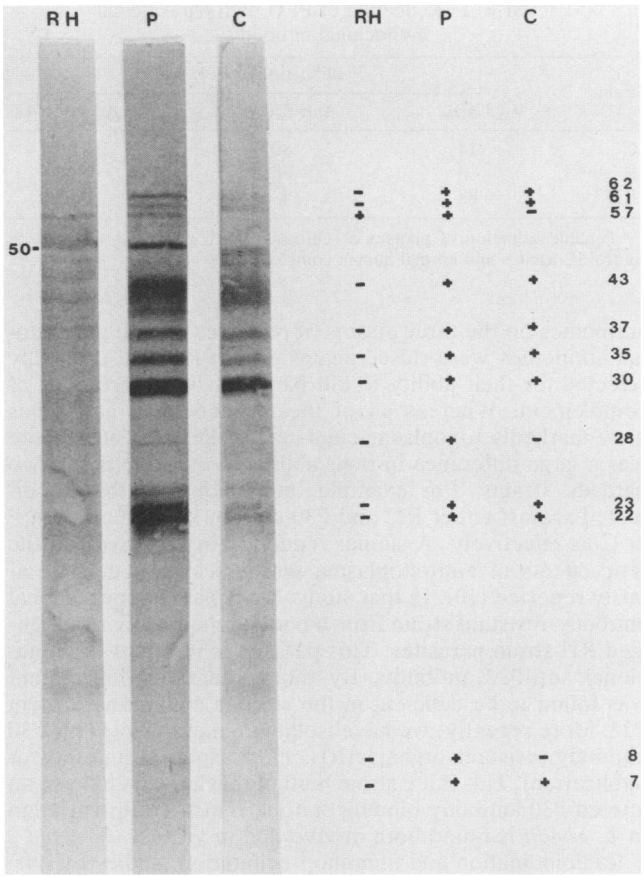

FIG. 4. Western blot analysis of RH, P, and C strain antigens reacted with strain-specific rabbit antisera. Equal numbers (3×10^6) of purified tachyzoites from strains RH, P, and C were subjected to 12.5% polyacrylamide gel and electrophoretically transferred to nitrocellulose paper, strips of which were incubated with corresponding antisera and assayed for IgM binding. The column of numbers on the left indicates antigens of the same molecular weight recognized by all three antisera. The column of numbers on the right indicates the molecular weights of unique or partially shared parasite antigens.

with the same results. Several antigens that shared by strains C and P were not detected by RH strain antiserum. These antigens had approximate molecular weights of 62,000, 37,000, 23,000, 8,000, and 7,000. There were also antigens that appeared to be unique to each of the three strains. These unique antigens had approximate molecular weights of 39,000 and 26,000 for strain RH, 54,000 for strain P, and 88,000 and 25,000 for strain C.

Figure 4 shows those antigens recognized by rabbit IgM antibodies. In general, fewer antigens were recognized by the IgM than the IgG fraction. Most striking was the reduced IgM response elicited by the RH strain. The only antigen that was detected on all three strains by both classes of antibodies was that with an approximate molecular weight of 50,000. The shared P and C strain antigens that were identified by the IgG fraction were also recognized by IgM. Note that strains C and P elicited an intense antibody response to P30 and P22, whereas strain RH failed to do so. An antigen of an approximate molecular weight of 57,000 shared by strains RH and P was not recognized by strain C antiserum. Also, a unique strain P antigen of an approximate molecular weight of 28,000 was not recognized by strain RH or C antiserum.

To show which parasite antigens are commonly shared and produce cross-reacting antibodies, Western blots with equal numbers (3×10^6) of parasites of each strain were reacted as a block with the same dilution (1:500) of each strain-specific or normal rabbit antiserum. Figure 5 shows those antigens recognized by the IgG fraction. Multiple antigens were detected on all three strains of parasites by each of the antisera. However, there were differences in the intensity of binding to parasite antigen by the various antisera. For example, P30 and P22 are commonly occurring antigens whose recognition by strain RH antiserum is quantitatively much less than that by strain P and C antisera. Interestingly, RH antiserum bound to a band at 62,000 molecular weight that appeared to be on strains P and C even though it was not expressed for an immunoblot of strain RH.

Figure 6 shows the commonly shared antigens identified by the IgM fraction. There was little difference between RH antiserum and normal rabbit serum. The single antigen possibly recognized by all three antisera had a molecular weight of 50,000 (P50). Strain C antiserum identified this antigen on all three strains, although with less intensity on strain RH. P antiserum also identified this antigen on all three strains but with the greatest intensity on strain P. RH antiserum only identified this antigen on strain RH. The antigen with an approximate molecular weight of 57,000 was recognized on both strains P and RH by RH antiserum, on all three strains by P antiserum, and not at all by strain C antiserum. Note that P and C strain-specific antisera recog-

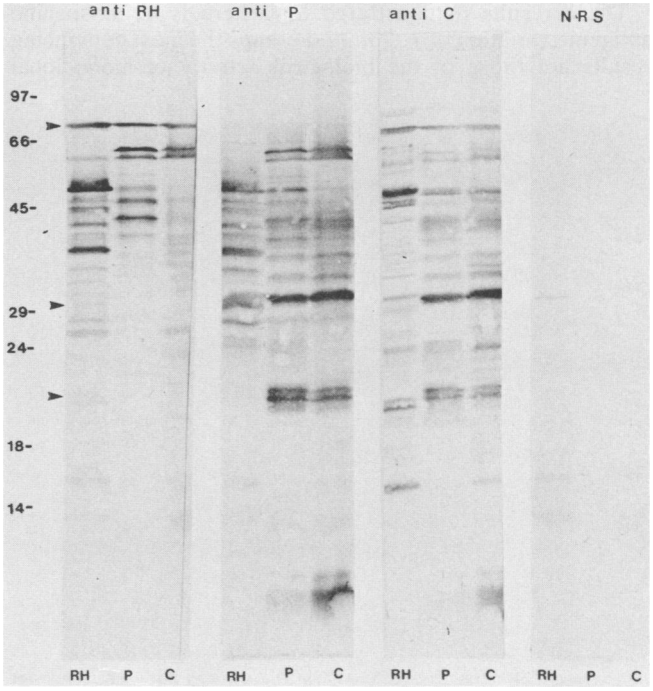

FIG. 5. Western blot analysis of common and cross-reacting RH, P, and C strain antigens identified by the IgG fraction of rabbit antisera. Equal numbers (3×10^6) of purified tachyzoites from strains RH, P, and C were subjected to 12.5% polyacrylamide gel and electrophoretically transferred to nitrocellulose paper. Numbers on left indicate molecular weight markers. Arrows indicate antigens that were detected on all three strains by each of the antisera. NRS, Normal rabbit serum.

nized P43, P30, and P22 on both P and C strains but failed to detect these antigens on strain RH.

Plaque reduction test. To support our findings of antigenic strain variability further, we used monoclonal antibodies raised against the surface membrane of *T. gondii*. We have previously reported the production of various murine hybridoma monoclonal antibodies to strain RH. Several of these monoclonal antibodies are parasiticidal in vitro in the presence of antibody-negative human serum. To determine whether these monoclonal antibodies were equally toxoplasmacidal to the three strains under investigation, we performed a plaque reduction test. We demonstrated that this assay is independent of parasite concentration and that no marked clumping of parasites occurs microscopically at high parasite concentrations. Each strain of parasite was treated with the three different monoclonal antibodies in the presence of antibody-negative human serum (Table 1). Monoclonal antibody 7B8 is directed against P30. By plaque reduction, 98% of strain RH, 80% of strain C, and 78% of strain P were killed. Another monoclonal antibody, 7G1, is directed against P22, a major radioiodinated membrane antigen of strain RH immunoprecipitated by human, rabbit, and mouse antisera. By this test, the number of plaques were reduced by 35% with strain RH, whereas neither C nor P strain plaques were reduced. Monoclonal antibody 12F12, which is presently under investigation in our laboratory, killed 88% of the RH parasites and 14% of strain C parasites and had no effect on strain P parasites. These results suggested that there is significant variation among the major antigenic determinants of the three strains under study.

DISCUSSION

These results demonstrated that there is strain-specific antigenic variation of *T. gondii*. Perhaps the most convincing results are those of the biological activity of monoclonal antibodies on the three strains of parasites. These monoclonal antibodies were raised against strain RH and originally selected for their ability to kill parasites in the presence of complement. Whereas all of these monoclonal antibodies were markedly toxoplasmacidal against RH parasites, there was a large difference in their abilities to kill the other two parasite strains. For example, monoclonal antibodies directed against either P22 and P30 did not kill either strain P or C as effectively. A similar reduction in sensitivity to the lytic effects of antitoxoplasma antibodies has been previously reported (10). In that study, we isolated a monoclonal antibody-resistant strain from a pool of chemically mutagenized RH strain parasites. This parasite is resistant to monoclonal anti-P22 antibody. By radioiodination, this mutant was found to be deficient in the surface membrane protein P22. More recently, we have isolated a monoclonal anti-P30 antibody-resistant organism (L. H. Kasper, submitted for publication). This is a P strain mutant that appears to have an altered P30 antibody binding epitope. Thus, strain variation in *T. gondii* is found both in vivo and in vitro.

TABLE 1. Reduction of PFU with parasiticidal monoclonal antibody

Strain	% Reduction of PFU with[a]:		
	12F12	Anti-P30 7B8	Anti-P22 7G1
C	14	80	0
P	0	78	0
RH	88	98	35

[a] Percent reduction of plaques as compared with parasites treated with control P3 ascites and normal human complement.

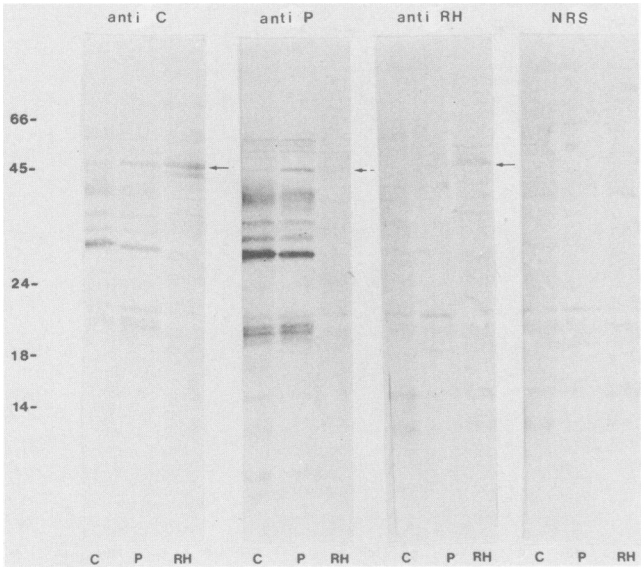

FIG. 6. Western blot analysis of common and cross-reacting RH, P, and C strain antigens identified by the IgM fraction of rabbit antisera. Equal numbers (3×10^6) of purified tachyzoites from strains RH, P, and C were subjected to 12.5% polyacrylamide gel and electrophoretically transferred to nitrocellulose paper. Arrows indicate the single antigen recognized by all three antisera. Numbers on the left indicate molecular weight markers. NRS, Normal rabbit serum.

Radioiodination and immunoprecipitation studies by ourselves and previous investigators have failed to show significant strain variation among the major membrane antigens of *T. gondii* (5, 6). There are, however, minor variations among these strains. In particular, an immunoprecipitable protein of molecular weight 40,000 (P40) is present in strains C and P but not in strain RH. Conversely, strain RH has two proteins of molecular weights 37,000 and 43,000 that are not apparent in the more naturally occurring C and P strain parasites. A more sensitive method for evaluating the antigenic nature of the three different strains of *T. gondii* is by Western blot analysis. Whereas there are a number of commonly shared antigens, as shown by the radioiodination and ELISA studies, there are quantitative and probably qualitative differences in the recognition of these shared antigens as detected by Western blotting. For example, P30 is a principal iodinated RH strain protein (Fig. 1). Monoclonal anti-P30 antibody raised against strain RH was highly lethal to RH parasites and significantly less so toward the other two strains studied. By Western blotting the immunogenicity of this protein, as well as that of the antiserum raised against it, differed among the three strains. Similar findings were also observed with another major RH protein, P22. As with P30, monoclonal anti-P22 antibody was parasiticidal against strain RH but had little effect against strain P or C.

There was also a striking difference in the IgM responses to the three different toxoplasma strains with immunoblot analysis. RH strain antigens were poorly reactive by this method, whereas both P and C strain antigens were readily identified. This difference between strains could be more clearly illustrated by RH strain P30, which could not be seen on IgM immunoblotting. However, a strong IgM response to P30 was revealed in both strains P and C by their corresponding antisera.

It is possible that these observations were due to different biochemical compositions of expressed membrane antigens. Proteins are iodinatable, whereas carbohydrates such as polysaccharides or the glycosylated residues of membrane surface proteins are not. Immunoprecipitation is done in the absence of reducing conditions and SDS. Western blot analysis requires these potentially protein-denaturing reagents. If the principle immunogenic component of an antigen is protein, then denaturation may occur during Western blotting. Alternatively, if the major antigenic determinant is glycosylated, then Western blotting will have little effect on its immunogenic character. Periodate treatment of glycosylated proteins (or lipids) will alter the carbohydrate structure and render it nonreactive. After periodate treatment of strain RH, Sharma et al. found a marked reduction in recognition by human antiserum (20). Thus, certain native antigens (i.e., P22 and P30) of the laboratory-maintained RH strain may have lost their glycosylated chains and only the core protein antigen persists after numerous serial passages. This hypothesis has been supported in part by previous investigators who have shown that RH strain *T. gondii* expresses low amounts of carbohydrates on its surface as determined by periodic acid staining and lectin binding (8, 15, 16, 19). We have observed a high affinity of strain C for a number of lectins (unpublished data). Continued passage of the parasite may result in progressive loss of these carbohydrates, giving rise to an increasingly virulent parasite that is finally unable to carry out its normal life cycle and undergo sexual differentiation in cat intestines. This alteration of parasite function with serial passage has been clearly demonstrated (2). We are presently investigating the carbohydrate structures of these three toxoplasma strains.

Our results demonstrated that antigenic strain variation in *T. gondii* occurs. Until now, we, as well as others, have failed to fully appreciate the significant immunogenic dissimilarities that may exist between the commonly used laboratory RH strain and the more naturally occurring, less virulent toxoplasma strains. Appreciation of these variants or subtypes may be important in the development of more sensitive diagnostic laboratory tests, as well as the long-range goal to produce a vaccine against human toxoplasmosis.

ACKNOWLEDGMENTS

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